Klinik für Kleintiermedizin
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Effects of hydrocortisone on systemic arterial blood pressure and urinary protein excretion and „white coat effect“ on blood pressure measurement in dogs

Artikel 1: Effect of long-term adaptation on indirect measurements of systolic blood pressure in conscious untrained beagles

Artikel 2: The Effects of Hydrocortisone on Systemic Arterial Blood Pressure and Urinary Protein Excretion in Dogs

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Abstract

Hypertension and proteinuria are commonly recognized in dogs with spontaneous hypercortisolism. There is, however, little information regarding the effect of exogenous glucocorticoids on blood pressure (BP) and proteinuria and whether these changes are reversible. Therefore the purpose of this study was to investigate if, how fast and to what degree experimental exogenous hypercortisolism results in hypertenison and proteinuria, and whether this changes are reversible.

Stress or anxiety associated with unknown people, an unfamiliar environment or the circumstances of the measurement, can result in a considerable increase in an animal's BP. Therefore, in a first part, BP measurement was repeatedly performed in dogs over several weeks to investigate if and how much adaptation to the measurement procedure influences the results. In a second part, BP, urine protein:creatinine ratio (UPC), microalbuminuria (MALB), urine albumin:creatinine ratio (UAC) and urine gel electrophoresis were evaluated in 12 Beagle dogs before, during and after administration of hydrocortisone (n=6, I-HC, 8mg/kg PO bid for 90 days) or placebo (n=6).

During the adaptation period, the BP decreased gradually and significantly, and levelled out after 14 days. The median (range) of values obtained by Doppler were 166 (149 to 200) mmHg initially, 145 (119 to 176) mmHg on day 9, 138 (118 to 165) mmHg on day 10, 127 (111 to 139) mmHg on day 35, 124 (115 to 143) mmHg on day 94 and 127 (114 to 142) mmHg on day 161. All the later measurements were significantly lower than the initial measurement. Male dogs had higher BP than females on each occasion.

In the second part, BP and UPC increased significantly during hydrocortisone administration from 123 mmHg (range 114-136 mmHg) and 0.17 (0.15-0.28) to a maximum of 143 mmHg (128-148 mmHg) and 0.38 (0.18-1.78), respectively on day 28. MALB developed in four dogs and UAC significantly increased in all dogs with the maximum on day 84. Both, BP elevation and proteinuria were reversible and completely resolved within one month after stopping hydrocortisone administration. SDS-AGE revealed the proteinuria to be primarily albuminuria with a pronounced increase during hydrocortisone treatment. Furthermore, a protein of 25-30 kDa was found in male dogs, identified by mass spectrometry to be arginine esterase, the major secretory prostatic protein.

In conclusion, measurements of blood pressure may be erroneously high in dogs not familiar with the measurement procedure. Therefore, in a clinical setting the measurement of an elevated blood pressure should be confirmed by repeating the measurement on at least two additional occasions before hypertension is diagnosed and antihypertensive treatment is started. Long-term hydrocortisone treatment, indeed, results in significant increases in systemic BP and urinary protein excretion. However, the changes are mild and in respect to BP, elevation to the point of systemic hypertension did not occur. Furthermore, all changes were reversible within one month after discontinuation of hydrocortisone.

Zusammenfassung

Der spontane Hyperkortisolismus beim Hund ist häufig mit Hypertension und Proteinurie assoziiert. Über den Effekt von exogenen Glukokortikoiden auf den Blutdruck, die Proteinurie und deren Reversibilität ist jedoch bislang nur wenig bekannt. Deshalb war das Ziel dieser Studie zu evaluieren ob, wie schnell und in welchem Masse experimentell-induzierter exogener Hyperkortisolismus zu Bluthochdruck und Proteinurie führt, und ob diese Veränderungen reversibel sind.

Stress oder Angst in Zusammenhang mit unbekannten Menschen, einer ungewohnten Umgebung oder der Blutdruckmessung an sich, können zu einem erheblichen Blutdruckanstieg führen. Um den Einfluss einer Adaptation an die Blutdruckmessung zu untersuchen, wurde in einem ersten Teil der Studie der Blutdruck über mehrere Wochen wiederholt gemessen. In einem zweiten Teil wurden bei 12 Beagle Hunden vor, während und nach Hydrokortisongabe (n=6, I-HC, 8mg/kg bid für 90 Tage) oder Placebobehandlung (n=6) der Blutdruck, der Protein/Kreatinin-Quotient (UPC), die Mikroalbuminurie (MALB), der Urin-Albumin/Kreatinin-Quotient (UAC) bestimmt und eine Uringelelektrophorese (SDS-AGE) durchgeführt.

Während der Adaptationszeit sank der Blutdruck graduell und signifikant ab und erreichte nach 14 Tagen ein Plateau. Die medianen (Bereich) Werte der Blutdruckmessung mittels Dopplertechnik lagen bei 166 (149-200) mmHg initial, 145 (119-176) mmHg am Tag 9, 138 (118-165) mmHg am Tag 10, 127 (111-139) mmHg am Tag 35, 124 (115-143) mmHg am Tag 94 und 127 (114-142) mmHg am Tag 161. Im Vergleich zur initialen Blutdruckmessung, waren alle folgenden Blutdruckmessungen signifikant tiefer. Männliche Hunde zeigten zu jedem Zeitpunkt einen höheren Blutdruck als weibliche

Im zweiten Teil der Studie zeigte der Blutdruck und das UPC während der Hydrokortisongabe einen signifikanten Anstieg von 123 (114-136) mmHg respektive 0.17 (0.15-0.28) auf ein Maximum von 143 (128-148) mmHg und 0.38 (0.18-1.78) am Tag 28. Eine MALB entwickelte sich in 4 Hunden und der UAC zeigte bei allen Hunden einen signifikanten Anstieg mit einem Maximum am Tag 84. Der Blutdruckanstieg und die Proteinurie waren innerhalb von einem Monat nach Absetzen der Hydrocortisongabe vollständig reversibel. Die SDS-AGE zeigte, dass die Proteinurie hauptsächlich aus einer Albuminurie besteht mit einem deutlichen Anstieg während der Hydrokortisongabe. Des Weiteren wurde ein Protein mit einem Molekulargewicht von 25-30 kD im Urin von männlichen Hunden gefunden, das mittels Massenspektrometrie als Argininesterase identifiziert wurde, das wichtigste sekretorische Protein der Prostata.

Zusammenfassend kann gesagt werden, dass Blutdruckmessungen bei Hunden, die nicht an die Blutdruckmessung gewöhnt sind falsch hoch sein können. Aus diesem Grund sollte bei klinischen Patienten ein erhöhter Blutdruck an mindestens 2 weiteren Tagen bestätigt werden, bevor die Diagnose einer Hypertension gestellt und eine Therapie begonnen wird. Eine Langzeit-Behandlung mit Hydrokortison führte zu einem signifikanten Blutdruckanstieg und einer erhöhten Proteinausscheidung über den Urin. Die Veränderungen waren nur leichtgradig und insbesondere der Blutdruck ist nicht auf ein hypertensives Niveau angestiegen. Alle Veränderungen waren innerhalb eines Monats nach Absetzen der Hydrokortisongabe reversibel.

Effect of long-term adaptation on indirect measurements of systolic blood pressure in conscious untrained beagles

S. SCHELLENBERG, T. M. GLAUS, C. E. REUSCH

To evaluate the effect of an adaptation period on systemic blood pressure readings, systolic blood pressure was measured in 12 young adult untrained beagles over several weeks by means of a Doppler flow detector and oscillometric devices. The pressure decreased gradually and significantly, and levelled out after 14 days. The median (range) of values obtained by Doppler were 166 (149 to 200) mmHg initially, 145 (119 to 176) mmHg on day 9, 138 (118 to 165) mmHg on day 10, 127 (111 to 139) mmHg on day 35, 124 (115 to 143) mmHg on day 94 and 127 (114 to 142) mmHg on day 161. All the later measurements were significantly lower than the initial measurement. Male dogs had higher blood pressures than females on each occasion. The blood pressure readings obtained with one of the oscillometric devices and the Doppler device were comparable and correlated significantly.

SYSTEMIC hypertension is a well recognised disorder in dogs, but in contrast to people, in whom primary 'essential' hypertension is most important (Kaplan 2001), hypertension in dogs most often appears to be secondary to an underlying disease, such as renal disease, hyperadrenocorticism, diabetes mellitus, pheochromocytoma, hyperaldosteronism and hypothyroidism (Breitschwerdt and others 1985, Gilson and others 1994, Bartges and others 1996, Ortega and others 1996, Struble and others 1998). A diagnosis of hypertension is based on the determination of systemic blood pressure. Although direct measurement is regarded as the gold standard (Kittleson and Olivier 1983), it is not usually feasible in practice and blood pressure is therefore measured by indirect methods. The Doppler flow detector and oscillometric devices have been validated in conscious dogs (Coulter and Keith 1984, Bodey and others 1996, Schneider 1999, Stepien and others 2003), but whichever device is used, blood pressure is a variable haemodynamic phenomenon that is influenced by many factors. Stress or anxiety associated with unknown people, an unfamiliar environment or the circumstances of the measurement, can result in a considerable increase in an animal's blood pressure (Belew and others 1999). This increase has been referred to as the white-coat effect and may lead to a false diagnosis of hypertension (Gosse and others 1993) and its unnecessary treatment. To minimise the effect it has been recommended that blood pressure should be measured in a quiet area and that the animal should be allowed to become acclimatised to its surroundings for at least five minutes. Furthermore, the first measurements should be discarded until the animal becomes accustomed to the procedure (Brown and Henik 1998). The importance of this short period of adaptation is well accepted, but little information is available about the long-term effects of adaptation. Remillard and others (1991) found that the diastolic, but not the systolic blood pressure of five healthy dogs decreased during a period of five weeks. The purpose of this study was therefore to evaluate the effect of long-term adaptation on indirect measurements of systolic blood pressure in conscious and untrained dogs.

MATERIALS AND METHODS

Twelve, three-year-old beagle dogs, six intact males and six intact females with bodyweights ranging from 10·4 to 16·6 kg (mean 13·2 kg) were used. They were considered to be healthy on the basis of the results of a physical examination, a complete blood count, a serum chemistry profile and urinalysis. The dogs had been kept in their housing facility away from the veterinary hospital since birth and supervised by the same caretakers for several months before the study began; they had been used regularly for student education, including clinical examinations, palpation and auscultation.

Experimental protocol

The blood pressure of each dog was measured on 12 occasions, on days 0, 9, 10, 14, 16, 17, 35, 67, 94, 147, 154 and 161. On days 0, 9, 10, 35, 94 and 161 it was measured by the Doppler method; on the other six occasions it was measured with an oscillometric device; on days 14, 16 and 17 using the Memoprint (S + B medVET) and on days 67, 147 and 154 using the SDI Vet/BP 6000 (SDI). To minimise the effect of the natural diurnal variability of blood pressure (Gelzer and Ball 1997, Piccione and others 2005), the measurements were made at the same time of day in each dog.

All the measurements were made by one person (S. S.) in the dogs' housing facility, with either an oscillometric device or an ultrasonic Doppler device (Model 811-B; Parks Medical Electronics). The dogs were placed in left lateral recumbency and allowed to acclimatise to their surroundings for at least five minutes before any measurements were taken. The first readings were discarded, and the arithmetic mean of five consecutive measurements was used for the data analysis.

For the oscillometric measurements an inflatable cuff was placed directly around the base of the dog's tail without clipping the hair (Vincent and others 1993). A cuff width of approximately 40 per cent of the circumference of the tail was chosen (Edwards 1990).

The Doppler ultrasonographic measurements were made by using the techniques described by Edwards (1990), Crowe and Spreng (1995) and Spreng and others (1996). The Doppler ultrasound probe was fixed in position over the superficial palmar arterial arch after the hair had been clipped and coupling gel applied. A cuff width of approximately 40 per cent of its circumference was placed around the mid-antebrachium. The cuff was inflated to 40 to 50 mmHg above the point at which the Doppler signal was no longer audible; the cuff was then slowly deflated and the systolic pressure was recorded as the pressure at which the pulse signal became audible.

Comparison of Doppler and oscillometric device measurements

The measurements were made in the 12 dogs with the Doppler device and SDI Vet/BP 6000 oscillometric device, using the

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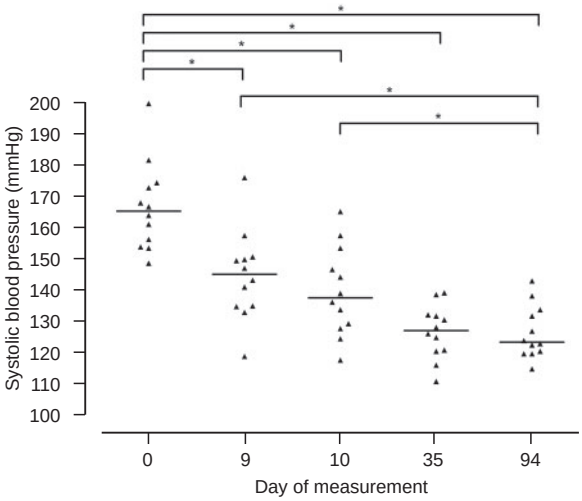

FIG 1: Median and individual measurements of systolic blood pressure made at intervals with the Doppler device in 12 conscious untrained beagles. *P<0·05

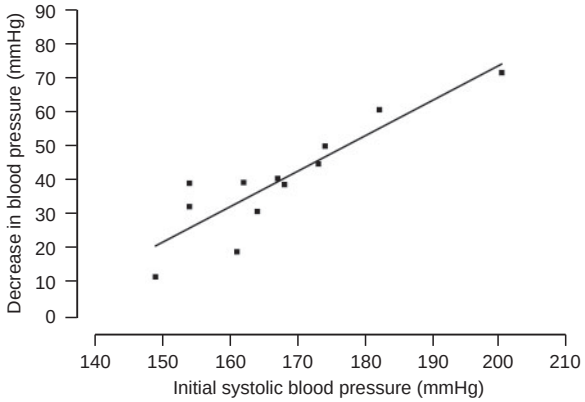

FIG 2: Relationship between the initial systolic blood pressure recorded in the 12 conscious, untrained beagles and the decrease observed after 161 days

same procedure as described above except that 10 minutes of acclimatisation preceded each series of measurements. After acclimatisation 10 oscillometric readings from the tail followed by five Doppler measurements from the forearm were obtained. The measurements were repeated nine times over a period of six months, during which time the dogs' systolic blood pressures measured by Doppler ranged between 96 and 164 mmHg.

Statistical analyses

The results were analysed by non-parametric statistical methods using SPSS 11.0 for Windows (SPSS). Median values and ranges were reported. The change in blood pressure during the adaptation period was analysed by means of Friedman's repeated measures test and a post hoc Wilcoxon test. For multiple comparisons, a Bonferroni correction was applied to the Wilcoxon test. Differences between male and female dogs were compared by the Mann-Whitney U test. The coefficient of variation was used as an estimate of the variability of the results. The measurements made oscillometrically were compared with the Doppler measurements by regression analysis and correlation coefficient, using GraphPad Prism 4 (GraphPad Software). The results of the two methods were also analysed according to Bland and Altman (1986). Differences were considered significant at values of P≤0·05.

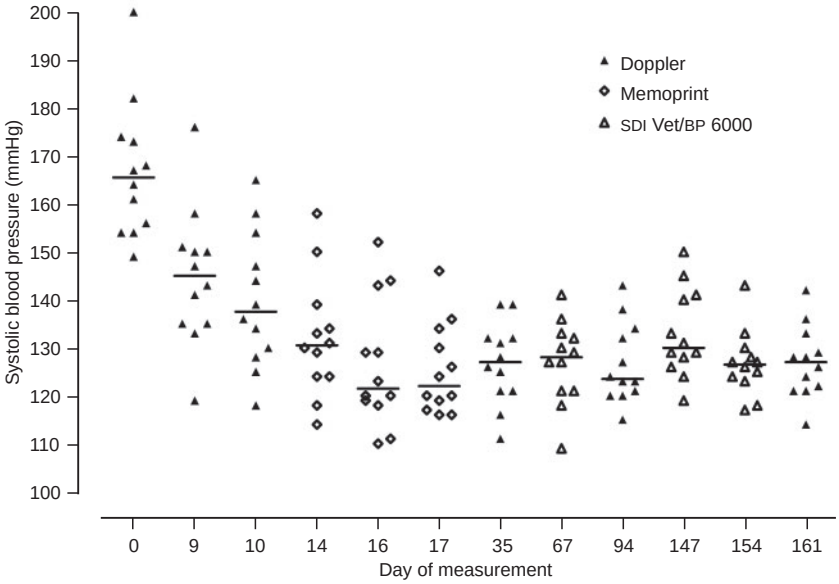

FIG 3: Measurements of systolic blood pressure made at intervals in 12 conscious, untrained beagles by Doppler, Memoprint and by SDI Vet/BP 6000

RESULTS

Effects of adaptation on blood pressure measured by Doppler

The systolic blood pressures measured by the Doppler method over a period of 94 days are shown in Fig 1. They showed a gradual and significant decrease over time. Initially eight of the 12 dogs had a systolic blood pressure of 161 mmHg or higher, but after nine days there had been a significant median decrease of 24 mmHg (range 4 to 40 mmHg) and there were further decreases after 10 and 35 days. After 94 days the pressure was significantly lower than after nine or 10 days. After the pressure had levelled out there was little variation in individual dogs from day to day, with a median coefficient of variation of 3·2 per cent (range 1·7 to 10·1 per cent). There was no significant difference between the mean blood pressure measured after 35, 94 and 161 days. The changes observed between the initial measurements and the measurements made 161 days later are shown in Fig 2. The dogs that initially had the highest systolic blood pressures had the largest decreases in blood pressure. The correlation coefficient of linear regression of the change in systolic blood pressures against the initial blood pressure was r=0·891 (P<0·0001).

There were initially wider variations in blood pressure between the dogs than there were later; initially, and after nine and 10 days the coefficients of variation were 8·9 per cent, 9·8 per cent and 10·1 per cent, respectively, compared with 6·7 per cent, 6·6 per cent and 5·8 per cent after 35, 94 and 161 days, respectively.

Effects of adaptation on the measurements

The measurements made with the three different devices are summarised in Fig 3. There was a steady reduction during the first 16 days, but thereafter the median systolic pressures were similar and fluctuated between 122 and 130 mmHg. During this period the measurements for each individual dog were reproducible with a coefficient of variation ranging from 1·9 to 8·0 per cent (median 4·2 per cent), although different methods of measurement were used.

Effects of sex

Throughout the study the male dogs had higher blood pressures than the females. The difference was not significant on individual days, but when all the measurements by the

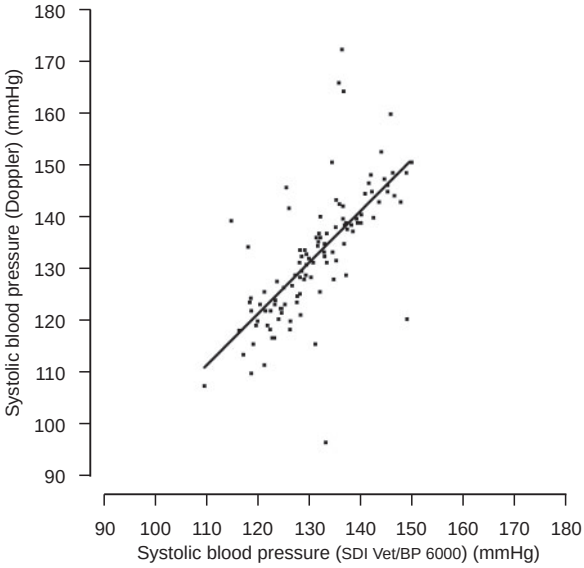

FIG 4: Relationship between the systolic blood pressures recorded in 12 conscious, untrained beagles by Doppler and by SDI Vet/BP 6000

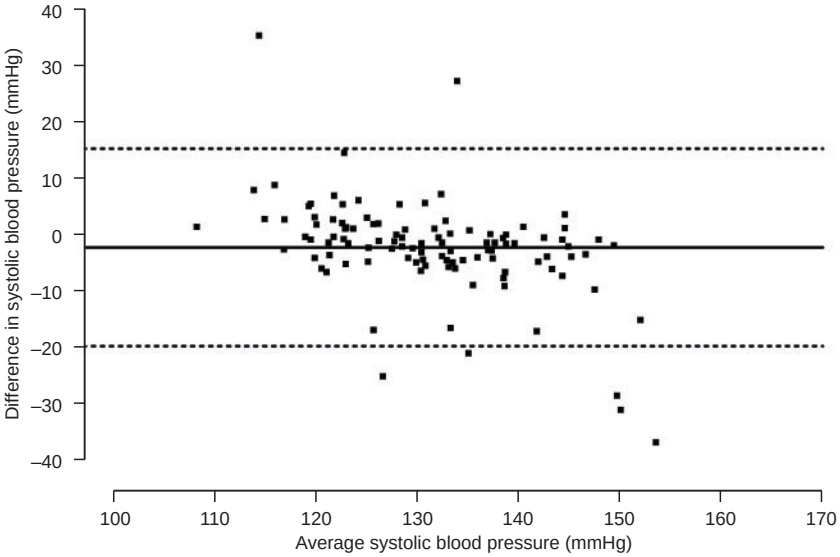

FIG 5: Bland-Altman plot of the difference between the measurements of systolic blood pressure in 12 conscious, untrained beagles made with SDI Vet/BP 6000 and the Doppler device and the average of their mean values. Solid line Mean difference, Dotted lines ±2 standard deviations

Doppler method were compared, the male dogs had significantly higher pressures (P=0·001).

Comparison of Doppler and oscillometric device measurements

A set of 108 measurements with the Doppler and the SDI Vet/BP 6000 were analysed. Linear regression revealed a significant correlation (P<0·0001) with a slope of 1·007 between the two methods (Fig 4). The same data, analysed by the method of Bland and Altman (1986) are shown in Fig 5. The oscillometric device bias was –1·6 mmHg, and the limits of agreement were ±17·8 mmHg; 69·4 per cent of the oscillometric device measurements were within 5 mmHg of the Doppler measurements and 88 per cent were within 10 mmHg.

DISCUSSION

For the diagnosis and treatment of systemic hypertension accurate and reliable blood pressure measurements are required. In people and dogs, it is well known that the act of measuring blood pressure leads to a rise in the systolic and diastolic blood pressure (Mancia and others 1983, Kallet and others 1997). This 'white-coat' effect develops at the beginning of a clinical evaluation and typically normalises within a few minutes (Mancia and others 1983). As a result, a short period of acclimatisation for five to 10 minutes is recommended to avoid the over-diagnosis and over-treatment of hypertension.

The results of this study show that in normotensive dogs previously unaccustomed to blood pressure measurements, their blood pressure was overestimated on the first few days and decreased significantly with repeated measurements on subsequent days. On the basis of their initial measurements eight of the 12 dogs satisfied conventional criteria for the diagnosis of hypertension, that is, a systolic pressure above 160 mmHg (Stepien and others 2003). However, on the second and third evaluation, only one dog, and on the following evaluations, no dogs, would have been classified as hypertensive. An erroneous diagnosis of hypertension would have been made despite applying the recommended acclimatisation period. Similar findings have been reported in human patients, whose blood pressure decreased after repeated measurements (Sinaiko and others 1989, Singh and others 1991). For this reason, in human medicine, at least three sets of readings at least a week apart should be obtained for a diagnosis of hypertension. If possible, the readings should be taken at home under varying conditions and at various times for at least four to six weeks with a semiautomatic device. If the diagnosis must be established more rapidly, a set of readings obtained with an automatic monitor over a period of 24 hours should be adequate (Kaplan 2001). In this study the dogs' blood pressure stabilised in a normotensive range between the fourth and fifth measurements, implying the importance of repeated measurements for a diagnosis of hypertension in dogs. After the dogs had become accustomed to the measurement procedure, their blood pressure did not change significantly even if the measurements were made after a long period of time.

The male dogs had a higher median blood pressure than females, in agreement with observations by Body and Michell (1996) in which entire male dogs had a higher blood pressure than neutered animals, and entire females had the lowest pressures. The reason is not understood, but thought to be due to a difference in temperament and a difference in sensitivity to stress.

This study was limited by not using the same method for measuring blood pressure on each occasion. The most pronounced decreases in pressure were observed at the beginning of the study, when only the Doppler device was used, but the comparison of the oscillometric and Doppler devices indicates that they give comparable readings under normotensive and mildly hypertensive conditions. The comparison of the Doppler and the Memoprint showed that there was good agreement between values in the normal range (110 to 160 mmHg), but only moderate or poor agreement in the hypotonic and hypertonic ranges (Schmieg 2002). During the study there were some outliers, with differences of up to 36 mmHg between the two methods, sometimes in the same dog (around 160 mmHg by Doppler and 130 mmHg by oscillometric device). These differences may have been due to real differences, because the measurements were not made simultaneously. It has been shown in dogs and people that large pressure fluctuations can occur, even within 60 seconds (Bovee 1993, Lossius and others 1993). The differences may also be associated with the different sites used to place the cuffs. However, there is always the potential for error when

a blood pressure measurement is obtained in a single individual, and the diagnosis of hypertension should always be made in relation to the clinical signs and any potential underlying disease.

Using research beagles instead of client-owned dogs may be another limitation of this study. Research beagles may be more nervous and excitable than other breeds, and may be less used to contact with different people and to manipulations. However, the beagles in this study had been living in their wards since birth, and had been handled by veterinarians for non-invasive procedures in the past. Thus, in some respects they may be considered to have been better adapted to veterinary manipulation than a client-owned dog presented to a clinic.

The results of this study indicate that measurements of blood pressure may be erroneously high in dogs not familiar with the measurement procedure. Repeated measurements on different days were associated with a significant decrease in pressure, and the decrease was greater when the initial pressures were higher. In a clinical setting blood pressure measurements should therefore be repeated on several days before hypertension is diagnosed and antihypertensive treatment is started.

References

BARTGES, J. W., WILLIS, A. M. & POLZIN, D. J. (1996) Hypertension and renal disease. *Veterinary Clinics of North America. Small Animal Practice* **26,** 1331-1345

BELEW, A. M., BARLETT, T. & BROWN, S. A. (1999) Evaluation of the white-coat effect in cats. *Journal of Veterinary Internal Medicine* **13,** 134-142

BLAND, J. M. & ALTMAN, D. G. (1986) Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* **i,** 307-310

BODEY, A. R. & MICHELL, A. R. (1996) Epidemiological study of blood pressure in domestic dogs. *Journal of Small Animal Practice* **37,** 116-125

BODEY, A. R., MICHELL, A. R., BOVEE, K. C., BURANAKURL, C. & GARG, T. (1996) Comparison of direct and indirect (oscillometric) measurement of arterial blood pressure in conscious dogs. *Research in Veterinary Science* **61,** 17-21

BOVEE, K. C. (1993) Genetic essential hypertension in dogs: a new animal model. In Veterinary Science – Growth Points and Comparative Medicine. Vol 4. The Advancement of Veterinary Science. Ed A. R. Michell. Wallingford, CAB International. pp 185-194

BREITSCHWERDT, E. B., MEUTEN, D. J., GREENFIELD, C. L., ANSON, L. W., COOK, C. S. & FULGHUM, R. E. (1985) Idiopathic hyperaldosteronism in a dog. *Journal of the American Veterinary Medical Association* **187,** 841-845

BROWN, S. A. & HENIK, R. A. (1998) Diagnosis and treatment of systemic hypertension. *Veterinary Clinics of North America. Small Animal Practice* **28,** 1481-1494

COULTER, D. B. & KEITH, J. C., Jr. (1984) Blood pressures obtained by indirect measurement in conscious dogs. *Journal of the American Veterinary Medical Association* **184,** 1375-1378

CROWE, D. T. & SPRENG, D. E. (1995) Doppler assessment of blood flow and pressure in surgical and critical care patients. In Kirk's Current Veterinary Therapy XII: Small Animal Practice. Ed J. D. Bonagura. Philadelphia, W. B. Saunders. pp 113-117

EDWARDS, N. J. (1990) Non-invasive blood pressure measurements in the clinical setting. Proceedings of the American College of Veterinary Internal Medicine 8th Annual Forum. Washington DC, USA, May 10 to 13, 1990. pp 273-276

GELZER, A. R. & BALL, H. A. (1997) Validation of a telemetry system for measurement of blood pressure, electrocardiogram and locomotor activity in beagle dogs. *Clinical and Experimental Hypertension* **19,** 1135-1160

GILSON, S. D., WITHROW, S. J., WHEELER, S. L. & TWEDT, D. C. (1994) Pheochromocytoma in 50 dogs. *Journal of Veterinary Internal Medicine* **8,** 228-232

GOSSE, P., PROMAX, H., DURANDET, P. & CLEMENTY, J. (1993) 'White coat' hypertension. No harm for the heart. *Hypertension* **22,** 766-770

KALLET, A. J., COWGILL, L. D. & KASS, P. H. (1997) Comparison of blood pressure measurements obtained in dogs by use of indirect oscillometry in a veterinary clinic versus at home. *Journal of the American Veterinary Medical Association* **210,** 651-654

KAPLAN, N. M. (2001) Hypertensive and atherosclerotic cardiovascular disease. In Heart Disease. 6th edn. Eds E. Braunwald, D. Zipes, P. Libby. Philadelphia, W. B. Saunders. pp 941-971

KITTLESON, M. D. & OLIVIER, N. B. (1983) Measurement of systemic arterial blood pressure. *Veterinary Clinics of North American Small Animal Practice* **13,** 321-336

LOSSIUS, K., ERIKSEN, M. & WALLOE, L. (1993) Fluctuations in blood flow to acral skin in humans: connection with heart rate and blood pressure variability. *Journal of Physiology* **460,** 641-655

MANCIA, G., BERTINIERI, G., GRASSI, G., PARATI, G., POMIDOSSI, G., FERRARI, A., GREGORINI, L. & ZANCHETTI, A. (1983) Effects of blood-pressure measurement by the doctor on patient's blood pressure and heart rate. *Lancet* **2,** 695-698

ORTEGA, T. M., FELDMAN, E. C., NELSON, R. W., WILLITS, N. & COWGILL, L. D. (1996) Systemic arterial blood pressure and urine protein/creatinine ratio in dogs with hyperadrenocorticisim. *Journal of the American Veterinary Medical Association* **209,** 1724-1729

PICCIONE, G., CAOLA, G. & REFINETTI, R. (2005) Daily rhythms of blood pressure, heart rate, and body temperature in fed and fasted male dogs. *Journal of Veterinary Medicine. A, Physiology, Pathology, Clinical Medicine* **52,** 377-381

REMILLARD, R. L., ROSS, J. N. & EDDY, J. B. (1991) Variance of indirect blood pressure measurements and prevalence of hypertension in clinically normal dogs. *American Journal of Veterinary Research* **52,** 561-565

SCHMIEG, L. F. (2002) Vergleich zweier indirekter Blutdruckmessmethoden an gesunden und kranken Hunden. Dissertation Ludwig-Maximilians-Universität München, Germany

SCHNEIDER, I. (1999) Vergleich der indirekten arteriellen Blutdruckmessung mittels zweier oszillometrisch messender Blutdruckmonitore mit der direkten Blutdruckmessung beim Hund. Dissertation Justus-Liebeg-Universität Giessen, Germany

SINAIKO, A. R., GOMEZ-MARIN, O. & PRINEAS, R. J. (1989) Prevalence of 'significant' hypertension in junior high school-aged children: the Children and Adolescent Blood Pressure Program. *Journal of Pediatrics* **114,** 664-669

SINGH, H. J., SINGH, R., SIRISINGHE, R. G. & UPADAYA, S. (1991) The variability of blood pressure following repeated measurements. *Medical Journal of Malaysia* **46,** 356-362

SPRENG, D. E., SPRENG, R., KIRBY, R. & SCHAWALDER, P. (1996) Monitoring of emergency and intensive care patients: simple diagnostic aids. *Schweizer Archiv für Tierheilkunde* **138,** 537-545

STEPIEN, R. L., RAPOPORT, G. S., HENIK, R. A., WENHOLZ, L. & THOMAS, C. B. (2003) Comparative diagnostic test characteristics of oscillometric and Doppler ultrasonographic methods in the detection of systolic hypertension in dogs. *Journal of Veterinary Internal Medicine* **17,** 65-72

STRUBLE, A. L., FELDMAN, E. C., NELSON, R. W. & KASS, P. H. (1998) Systemic hypertension and proteinuria in dogs with diabetes mellitus. *Journal of the American Veterinary Medical Association* **213,** 822-825

VINCENT, I. C., MICHELL, A. R. & LEAHY, R. A. (1993) Non-invasive measurement of arterial blood pressure in dogs: a potential indicator for the identification of stress. *Research in Veterinary Science* **54,** 195-201

The effects of hydrocortisone on systemic arterial blood pressure and urinary protein excretion in dogs

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THE EFFECTS OF HYDROCORTISONE ON SYSTEMIC ARTERIAL BLOOD PRESSURE AND URINARY PROTEIN EXCRETION IN DOGS

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Short title: Blood Pressure and Proteinuria in Hypercortisolism

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ABSTRACT

Background: Hypertension and proteinuria are commonly recognized in dogs with spontaneous hypercortisolism. There is, however, little information regarding the effect of exogenous glucocorticoids on blood pressure (BP) and proteinuria and whether these changes are reversible.

Hypothesis: Hydrocortisone administration increases systemic BP and urinary protein excretion, and these effects are reversible after hydrocortisone withdrawal.

Animals: 6 control dogs and 6 dogs treated with hydrocortisone.

Methods: BP, urine protein:creatinine ratio (UPC), microalbuminuria (MALB), urine albumin:creatinine ratio (UAC) and urine gel electrophoresis were evaluated before, during and after administration of hydrocortisone (8mg/kg p.o. q12h for 12 weeks) or placebo.

Results: BP and UPC increased significantly during hydrocortisone administration from 123 mmHg (range 114-136 mmHg) and 0.17 (0.15-0.28) to a maximum of 143 mmHg (128-148 mmHg) and 0.38 (0.18-1.78), respectively on day 28. MALB developed in four dogs and UAC significantly increased in all dogs during hydrocortisone administration with the maximum on day 84 (0.133 (0.034-1.251)). Both, BP elevation and proteinuria were reversible and completely resolved within one month after stopping hydrocortisone administration. SDS-AGE revealed the proteinuria to be primarily albuminuria with a pronounced increase during hydrocortisone treatment. Furthermore, a protein of 25-30 kDa was found in male dogs, identified by mass spectrometry to be arginine esterase, the major secretory prostatic protein.

Conclusions and Clinical Importance: Long-term hydrocortisone treatment results in significant but only mild increases in systemic BP and urinary protein excretion, which are both reversible within one month after discontinuation of hydrocortisone.

Key words: Hypercortisolism, Hypertension, Proteinuria, Albuminuria, Arginine Esterase

INTRODUCTION

Hypertension is a frequent finding in humans and dogs with exogenous or endogenous glucocorticoid excess. The prevalence of systemic hypertension in human patients with spontaneous (endogenous) hypercortisolism (HC) has been reported to be approximately 80%.[1] In experimental studies in humans, glucocorticoids have been shown to increase systolic (SAP) as well as diastolic (DAP) arterial blood pressure.[2,3] Significant increases in SAP were identified within 24 hours of cortisol administration, but iatrogenic glucocorticoid-induced hypertension appears to be less severe than in the natural disease.[2] The prevalence of hypertension in dogs with spontaneous HC is similar at 59-86%.[4,5] When treating dogs with spontaneous HC, adequate suppression of cortisol secretion results in decreasing blood pressure (BP), however, hypertension is still common in the population of well controlled HC.[4] In contrast, in experimental dogs treated with hydrocortisone for several weeks, SAP was not significantly higher compared to placebo treated dogs.[6]

Proteinuria is also commonly observed in dogs with HC. The incidence of pathological proteinuria, defined as a urine protein:creatinine ratio (UPC) greater than 1.0, has been reported to be 44 - 75%.[4,7] Experimentally, longterm administration of prednisolone resulted in proteinuria of glomerular origin.[8] The reversibility of proteinuria in spontaneous HC is controversial. Whereas Ortega et al. found a significant decrease in UPC in dogs with well-controlled HC, Hurley and Vaden did not.[4,7]

Much of the existing information about the effects of glucocorticoids on blood pressure and proteinuria has been generated by experimental studies that used prednisolone for the induction of proteinuria and hypertension. However, in spontaneous HC the endogenous glucocorticoid hydrocortisone seems to be the major cause of clinical and laboratory abnormalities.[9]

In view of the paucity of knowledge on the effect of hydrocortisone on systemic BP, urinary protein excretion, and particularly the reversibility of such changes in dogs, the purpose of this study was to induce experimental HC using hydrocortisone as an attempt to most closely mimic the natural disease. The specific goals then were first to assess the developement and severity of hypertension, second quantitating and characterizing the glucocorticoid induced proteinuria, third to assess the association between urinary protein excretion and BP, and fourth, to study the reversibility of these changes.

MATERIAL AND METHODS

This randomized, placebo-controlled study was approved by the Cantonal Veterinary Office (Zurich, Canton of Zurich, Switzerland).

Dogs

The study was conducted using twelve 3.5-year old Beagle dogs, 6 intact males and 6 intact females, with a body weight ranging from 10.4 to 16.6 kg (median 12.9 kg). The dogs were determined to be healthy on the basis of physical examination, CBC, serum biochemistry profile, urinalysis, urine culture, UPC ratio and indirect BP measurement.

Study Design

HC was induced by hydrocortisone, the synthetic glucocorticoid most similar to cortisol. The dosage was chosen extrapolating from previous studies inducing I-HC in dogs.[6,8] Dogs were randomly allocated to two groups of 6 dogs. Dogs in the control group received a placebo gelatin capsule PO q12h while dogs in the treatment group received hydrocortisone[a] at a median dose of 8.5 mg/kg (range of 7.5-9.6 mg/kg) PO q 12h for 84 days (I-HC group). Dogs were examined before (d0), and 1 (d1), 5 (d5), 28 (d28), 56 (d56), 84 (d84) days after starting treatment and 1

(d1p), 5 (d5p), 28 (d28p), 56 (d56p) and 84 (d84p) days after withdrawal of hydrocortisone and placebo, respectively. Dogs were observed for typical cortisol-induced clinical abnormalities including PU/PD and hair loss , but only subjectively, i.e. water intake and urine production were not quantitated and it was not attempted to use a scoring system for the skin abnormalities. On d0, d28, d56, d84, and d1p an ACTH stimulation test was performed in all dogs by obtaining samples for determination of cortisol before and 1 hour after IM injection of 0.25 mg of synthetic ACTH[b]. Cortisol concentrations were determined by use of a previously validated chemiluminescence method (ADVIA Centaur® System, Bayer (Schweiz) AG, Zurich, Switzerland).[10]

Blood pressure measurement

Before the beginning of the study, dogs were acclimated to the blood pressure measurement procedure on 12 different days, to minimize excitement and anxiety during the study.[c] Systolic (SAP), diastolic (DAP) and mean (MAP) arterial pressure were measured using an indirect oscillometric device[d]. An inflatable cuff of approximately 40% of the tail circumference was placed directly around the base of the tail without clipping hair.[11] Before recording BP, dogs were placed in left lateral recumbency and allowed to acclimate to the surroundings for at least 10 minutes and the first BP readings were discarded. For data analysis the arithmetic mean of 10 measurements was used.

Urinary Samples

Urine samples were collected from each dog by use of ultrasound-guided cystocentesis for routine urinalysis, urine culture, UPC, and for assessing microalbuminuria, urine albumin:creatinine ratio (UAC) and urine protein electrophoresis.

Assessment of proteinuria

Urine protein:creatinine ratio

Total urine protein levels were assayed on an autoanalyzer by an immunoturbidimetric method. Urine creatinine concentrations were determined using a commercial autoanalyzer-based kinetic Jaffe reaction and UPC ratios were calculated.

Microalbuminuria and urine albumin:creatinine ratio

Urine albumin concentration was determined by two different methods. Microalbuminuria (MALB) was assayed semiquantitatively by means of the canine E.R.D.-Health Screen test[e] after urine samples were normalized to a urine-specific gravity of 1.010 to allow comparison among samples. The amount is indicated as none (negative), small (+), moderate (++), and high (+++). To quantitate albuminuria, a modified human immunoturbidimetric assay[f] validated for the dog was used as described previously[12], and UAC ratios were calculated. To assess sensitivity and specificity of the MALB, 2 different UAC cut-offs were chosen, first the mean+2SD obtained in our control dogs, second 0.03, the cut-off for pathologic microalbuminuria in people.[13]

Urine protein electrophoresis

Urinary proteins were separated by sodium dodecyl sulfate agarose gel electropheresis (SDS-AGE) using the Hydragel proteinurie kit[g].[14] Briefly, twenty microliters of a 10 g/L SDS solution were added to 80 µL of urine. Five microliters were loaded to each well (5 wells/gel) and submitted to electrophoresis in an SDS-imidazole pH 7.0 buffer. After the gel was completely dried at 80 °C for 20 min, it was immersed for 30 min in an aqueous staining solution (acid violet) and destained in two successive aqueous baths before it was immersed in a glycerol aqueous solution and dried at 80°C (15 min).

Characterization of the male specific protein

Gel protein bands were cut with a scalpel and repeatedly washed in 25 mM NH_4HCO_3 with subsequent shrinking of the gel pieces through addition of 50 % acetonitrile in 25 mM NH_4HCO_3. The proteins were digested overnight by adding 19 ng/µl sequencing grade modified trypsin[h] in 47mM Tris pH 9. Peptides were extracted from the gel by sonification in buffer A (2 % acetonitrile, 0.1 % trifluoroacetic acid). For liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS/MS) analysis the protein supernatant was run over a C18 column eluting the peptides at 600 nl/min in 45 min by using a gradient of 95 % buffer A to 60 % buffer B (80 % acetonitrile, 0.1 % trifluoroacetic acid). Peptides were ionized using electro spray ionization and directly injected into the massspectrometer LCQ Deca XP[i] recording their mass-to-charge ratio. Following each full MS spectrum the two most intense peaks were automatically selected for fragmentation of these peptides and recording of the MSMS signals (fragments). All MSMS spectra were submitted to Mascot[15] for identification of the proteins.

Statistical analysis

Results were analyzed by use of nonparametric statistical methods[j,k], and reported as median and ranges. Differences within one group were tested by use of Friedman's repeated measures test followed by Dunn's post-tests. Differences between the groups were tested by use of the Mann-Whitney U test. Differences were considered significant at values of $p \leq 0.05$.

RESULTS

Induction of HC

In this model, long-term hydrocortisone administration effectively created hypercortisolism. All dogs receiving hydrocortisone developed clinical signs (polyuria, polydipsia, no regrowth of clipped hair within one month, and thinning of the ventral abdominal skin with prominent

subcutaneous veins), and laboratory abnormalities (stress leukogram, increased alkaline phosphatase activity, isosthenuria) consistent with cortisol excess. During medication dogs in the I-HC group had significantly higher baseline plasma cortisol concentrations than dogs in the control group (e.g. d56: 3 hours post pill 30.7 mcg/dl (13.3-57.6) versus 0.5 mcg/dl (0.1-2.5)). Finally, 36 hours after hydrocortisone withdrawal dogs in the I-HC group failed to respond to ACTH stimulation; median plasma cortisol concentrations before and after ACTH administration were 0.25 mcg/dl (0.1 – 1.4 mcg/dl) and 0.45 mcg/dl (0.1 - 2 mcg/dl), respectively.

Blood pressure

Initially, median SAP, DAP and MAP were 128 mmHg (122 - 142 mmHg), 63 mmHg (55 - 83 mmHg), and 90 mmHg (78 - 104 mmHg) in the control group and 123 mmHg (114 - 136 mmHg), 69 mmHg (53 - 78 mmHg), and 90 mmHg (81 - 101 mmHg) in the I-HC group, respectively. Hydrocortisone but not placebo treatment resulted in a progressively increasing SAP, DAP and MAP that peaked on day 28 (Table 1). The increase in SAP above baseline was significant on day 28 ($p < 0.01$) and 56 ($p < 0.05$), but not anymore on d84. Elevations of DAP and MAP were significant on d28, d56 and d84. SAP, DAP and MAP decreased to baseline values within the first 5 days after hydrocortisone withdrawal, and did not change over the following 11 weeks (Table 1).

Proteinuria

Urine protein:creatinine ratio

Initially, UPC was below 0.4 in all dogs (0.17; 0.13-0.38). No change in UPC was seen in control dogs throughout the study (Table 2). In the I-HC group, UPC ratios increased significantly ($p < 0.0001$) during hydrocortisone administration to a peak on day 28. UPC ratios on day 28 and day 84 were significantly higher compared to day 1 and 2 (Fig. 1). Compared to

the control group UPC ratios in the I-HC group were significantly higher on day 28 and 56. Only two out of six dogs in the hydrocortisone group developed clinically significant proteinuria defined as UPC ratio > 0.5[16], with maximal values in these two dogs of 1.78 and 1.63. One of these dogs (UPC ratio 1.78) showed pyuria with 8-12 leucocytes, results of urine cultures were negative in both these (and all the other dogs throughout the study), and urine electrophoresis showed a strong band in the molecular weight range of 65-70 kDa corresponding to albumin in both dogs. On d84 the dog with pyuria again showed an increased UPC ratio of 1.63, this time without pyuria, a negative urine culture and still a single band in the molecular weight range of 65-70 kDa.

In both groups there was a significant, but weak correlation between UPC ratios and SAP ($r = 0.611$, $p = 0.0001$ in control dogs and $r = 0.634$, $p = 0.0005$ in the I-HC group). After hydrocortisone withdrawal UPC gradually decreased, reaching pre-treatment values within one month.

Male dogs consistently had a higher UPC ratio than female dogs, except on d28, d56 and d84 under hydrocortisone treatment, i.e. at times when urinary protein excretion was affected by hydrocortisone administration (Table 2).

Microalbuminuria and urine albumin:creatinine ratio

Before starting treatment all dogs in the I-HC group were found to be negative for MALB, whereas 2 male dogs in the control group were positive (++). During treatment 4 dogs in the I-HC group developed variable degrees of MALB (+, ++, or +++) on days 28, 56 and 84, but not consistently. One month after stopping hydrocortisone administration MALB was no longer detectable in these 4 dogs. One dog that was negative for MALB during hydrocortisone administration was positive (++/+) on day 1 and 5 after hydrocortisone withdrawal. The two

dogs in the control group that were positive before the start of treatment remained positive, and all other control dogs remained negative throughout the study.

UAC ratios before treatment were 0.017 (0.005 – 0.082) in the control group and 0.015 (0.009 – 0.023) in the I-HC group. No change in UAC was seen in control dogs throughout the study (Table 2). UAC ratios gradually increased during hydrocortisone administration (Fig. 2) reaching a maximum on day 84, which showed a 13-fold (2.5 – 73.6) increase. UAC ratios were significantly higher on day 84 compared to baseline and day 1, and on day 56 compared to day 1. After stopping hydrocortisone administration UAC progressively decreased and returned to baseline values within 28 days. UAC ratios in the two control dogs which were positive in the MALB test throughout the study, ranged between 0.044 and 0.098 (median 0.067), and 0.048 and 0.113 (median 0.081), respectively.

Correlation between the microalbuminuria test and urine albumin:creatinine ratio

There was a moderate and significant correlation between UAC and the MALB concentrations ($r = 0.719$, $p < 0.0001$). Overall 105 samples had a negative MALB result, 31 had a + result, four a ++, and four a +++ result. UAC ratios in the different categories of MALB were 0.002-0.131 (median 0.012) for negative, 0.044-0.242 (0.079) for low positive, 0.164-0.725 (0.325) for medium positive and 0.242-1.251 (1.023) for strong positive results. At a UAC cut-off of 0.078 (mean+2SD in control dogs), sensitivity and specificity of the MALB were 81% and 88%, respectively. At a UAC cut-off of 0.03, sensitivity and specificity of the MALB were 64% and 100%, respectively.

Urine protein electrophoresis

In the urine of the dogs two distinct bands were found by SDS-AGE, one in the molecular weight range of 65-70 kDa corresponding to albumin and the other in the range of 25-30 kDa. The

patterns in these dogs were characterized by either no band, an isolated band of 65-70 kDa, an isolated band of 25-30 kDa or both bands (Fig. 3A). Each dog of the I-HC group developed a band or showed a pronounced increase in the density of the band in the range of 65-70 kDa. After stopping hydrocortisone administration, the intensity of the albumin band decreased starting on day 5 and patterns were similar to baseline in each dog at d28p. A representative example of an SDS-AGE for one of these dogs is depicted in Fig. 3B.

The band in the range of 25-30 kDa was only present in urine samples from male dogs. Each male dog showed this band during the study period, but not on every occasion. The sequence analysis of the tryptic peptides by LC-MSMS resulted in a sequence coverage of 6-16%. The partial sequences AVIRPGEDR (Fig. 4) and SHDLMLLHLEEPAK of canine arginine esterase were sequenced in all tested protein bands with a Mascot significance threshold of 0.05 searching against the full Uniprot database. In some of the samples MSMS spectra matching the peptides SFIHPLYK and VMPHLMWIK were found. There was no additional significant Mascot hit for another protein in this band.

DISCUSSION

Hydrocortisone administration consistently increased BP in the dogs in this study. Whereas in humans a significant rise in SBP has been described within as little as 24 hours of commencing cortisol administration[2,17] the rise in our dogs was not significant on days 1 and 5, but only on day 28. In contrast to dogs with spontaneous HC, where hypertension is a common finding and may be marked[18], no dog in our study developed increased BP to the point of hypertension defined as SAP > 160 mmHg.[19] As a matter of fact, the increase in SAP averaged only 20 mmHg (12-21 mmHg) within 1-2 months of hydrocortisone administration and thereafter even tended to

decrease during the treatment period. Recently, categorization of BP has been recommended due to risk of developing subsequent target organ damage. Applying this new classification scheme of hypertension, all dogs stayed in the lowest risk category (BP < 150/95 mmHg).[20]

This discrepancy may have different causes. First, glucocorticoids may not be a relevant contributor to hypertension in spontaneous HC, second, the prevalence of hypertension in spontaneous HC may have been overestimated and third, our model may not closely resemble spontaneous HC. In dogs with spontaneous HC hormones other than cortisol may be responsible for the development of hypertension. Although in HC hypercortisolemia is considered to be of primary pathophysiological importance in dogs, excess production of aldosterone and precursors of the glucocorticoid and mineralocorticoid pathways, i.e. 17a-OH-pregnenolone, 17a-OH-progesterone, 11-deoxycortisol, 21-deoxycortisol and corticosterone have been reported.[10,21,22] Of these, certainly aldosterone, but also various precursors may play a role in the development of hypertension.[23,24] BP and the prevalence of hypertension may have been overestimated in clinical studies on HC. It has been shown that BP values strongly depend on acclimation to the measurement procedure[25,26], with decreasing values over several days of measurements. Therefore, if BP measurements are obtained on a single day only, false high readings may result and hypertension may be overdiagnosed. A limitation of our model may be that our experimental dogs were only 3.5 years old, whereas spontaneous HC is typically a disease of middle-aged to older dogs. BP has been shown to increase with age[27]; thus, confounding factors related to age, e.g. atherosclerosis and obesity, may play a role in developing hypertension in spontaneous HC, whereas in our experimental healthy dogs excessive hydrocortisone was the only variable. Also, in spontaneous HC the cortisol excess may be more gradual but present for a much longer time. In this respect it is interesting to note, however, that blood pressure did not continue to increase

277 in the third month of hydrocortisone administration but tended, rather, to decrease, suggesting
278 that there is no linear relationship between duration of HC and increasing blood pressure.
279 Finally, with administration of hydrocortisone twice a day we created only two plasma peaks of
280 cortisol, whereas in spontaneous HC there are multiple peaks throughout the day.[28]
281 As indicated, not only was the increase in BP mild, but also SAP again started to decrease after
282 d28. An explanation for this finding may be that chronic glucocorticoid treatment results in some
283 metabolic adaptation. Glucocorticoid receptor downregulation in reaction to GC therapy was
284 demonstrated in vitro and in vivo,[29,30] but the possible mechanism of this receptor
285 downregulation is poorly understood. There is evidence for an enhanced receptor degradation
286 and a modulated GR expression.[31,32] A recent study showed that in humans with endogenous HC
287 not a GR downregulation but a diminished ligand affinity might partially protect the cells from
288 the high cortisol levels.[33] Another explanation for this finding may be that measurement on day
289 56 and 84 fell into the months of July and August, i.e. the hottest months of the year. High
290 temperatures result in vasodilatation, decreasing peripheral vascular resistance and reduction in
291 BP.[34] Also, extracellular volume due to water loss by panting and impaired urine concentrating
292 ability may have been lower in dogs with HC. However, all dogs in the study had free access to
293 water and a cool environment, and hematocrit was not different over time between groups, thus
294 there was no reason to suspect hypovolemia.
295 In all our experimental dogs BP returned to basal values shortly after cessation of hydrocortisone
296 administration. In contrast, in dogs and humans with spontaneous HC hypertension often persists
297 despite appropriate treatment and improvement of other clinical signs.[4,35,36] This again indicates
298 that hypertension in spontaneous HC is not just caused by the cortisol excess per se. The
299 persistence of hypertension in spontaneous HC could be the result of irreversible peripheral

vascular remodeling followed by increased wall stiffness and increased vascular resistance. This hypothesis is supported by the finding that patients with a history of hypertension of less than 5 years were more likely to become normotensive after adrenalectomy.[37] However, the factors that influence persistence of hypertension have not been studied in either humans or dogs.

In addition to an elevated BP we observed increased urinary protein excretion in hydrocortisone treated dogs. The increases in UPC ratios in most dogs were mild and smaller compared to dogs with spontaneous HC[4] or dogs receiving prednisone for 42 days.[8] The increase in proteinuria is thought to be primarily of glomerular origin, as there was only albuminuria as determined by SDS-AGE. Nevertheless, proteins other than albumin that may have been in the urine of our dogs were lost because the increase in UAC does not account for the increase in UPC, i.e. UPC was higher than UAC; thus, SDS-AGE was not sensitive enough to detect small amounts of multiple various proteins.

Possible causes of increased glomerular proteinuria include increased intraglomerular pressure and damage to the glomerular basement membrane. Elevated BP and a significant albeit weak positive correlation between SAP and UPC in our dogs supports hypertension as being one causative mechanism of proteinuria. Even though systemic hypertension does not directly translate into increased intraglomerular pressure, glucocorticoids have also been shown to increase renal plasma flow and glomerular filtration rate in dogs.[38,39,40] Thus, glucocorticoids may mediate proteinuria by hemodynamic alterations resulting in an increase in glomerular pressure. Altered renal histomorphology was found in dogs treated with prednisone for 42 days.[8] The most consistent finding was mild to moderate hypercellular glomerular tufts, suggestive of mesangial proliferation. Other findings were glomerular adhesions and moderately thickened Bowman's capsules. Electron microscopy was characterized by occasional mild segmental

thickening of basement membranes, fusion of visceral cell foot processes and glomerular adhesions. Lack of pre-treatment renal histologic examination, lack of a control group and lack of follow-up histologic examination after discontinuation of glucocorticoids makes it difficult to say whether these changes are a result of glucocorticoid administration and whether histologic changes are reversible. As in our study no renal histologic examinations were performed, our results cannot answer the question of whether the quickly reversible proteinuria was due to either functional or morphological changes, or both. In spontaneous HC, proteinuria may or may not significantly decrease when the disease is well controlled.[4,7,41] Presumably, elevated intraglomerular pressure in HC will lead to reversible or irreversible morphologic changes, depending on degree and chronicity.

The 17% prevalence (2/12) of MALB in the healthy dogs in this study before any treatment is in accordance with other studies reporting a prevalence in healthy dogs ranging between 15 and 19%.[42,l] It is important to note that there may be significant day-to-day variations of up to 30 - 80% in daily urinary albumin excretion in man.[43] This variation is dependent on posture, exercise, and dietary factors, such as protein intake.[44,45] Data from human patients indicate that MALB is associated with an increased cardiovascular risk in hypertensive patients[46,47] and the nondiabetic population.[48,49] In our two healthy dogs follow-up assays were positive throughout the study, and they are therefore thought to be microalbuminuric, however, the significance of this finding is unknown. The most important consideration when assessing MALB should probably be the context of where this result is obtained, i.e. is it any patient or a patient at risk of end organ damage. In this respect, the authors are unaware of any data that associate MALB with increased morbidity or mortality in dogs.

MALB can be quantitated by different means. Enzyme-linked immunoassay analysis, nephelometry and immunoturbidimetry are complicated, time consuming and expensive laboratory techniques, unsuitable for routine use. Recently, the ERD HealthScreen® test, a simple and fast test for the semiquantitative detection of MALB has been commercialized. In the present study, to verify the accuracy of MALB observed in healthy dogs and in dogs with iatrogenic HC as measured by this simple screening test we performed immunoturbidimetry as a quantitative reference method. In human medicine, an UAC ratio > 0.03 is considered abnormal and indicative of pathological MALB.[13] Unfortunately few data exist on the normal range of UAC in healthy dogs, but in a previous study mean UAC ratio in 10 healthy dogs was 0.04±0.08[m]. The two healthy dogs with MALB were several times above the level of 0.078 and thus correctly identified with the screening test. Based on our findings the ERD HealthScreen® test appears to be an efficient and fast method for the semiquantititative detection of MALB in dogs with good correlation to a quantitative laboratory method.

Higher UPC in male dogs when urinary protein excretion is not affected by hydrocortisone administration is explained by the distinct protein band in the molecular weight range of 25 to 30 kDa, identified as arginine esterase. This enzyme, the major secretory protein of the prostatic gland, enters the bladder by reflux from the proximal urethra.[50,51] An isolated band in the same molecular weight range has recently been described as free light chains and linked to male *and* female dogs exposed to or infected with certain infectious agents; i.e. Leishmania infantum, Ehrlichia canis and Babesia canis.[52] On the basis of our results, this protein band is a physiological finding in urine samples of intact male dogs, i.e. argininge esterase, and does not necessarily represent free light chains in every case.

In conclusion, our study showed that long-term hydrocortisone administration induces mild elevation of BP and mild proteinuria mainly characterized by albuminuria, suggesting proteinuria of glomerular origin. Both hypertension and proteinuria were reversible and completely restored within one month after cessation hydrocortisone administration. The higher UPC ratio in intact male dogs compared to female dogs can be explained by the physiological presence of arginine esterase.

[a] Hydrocortisone tablets, Hotz Pharmacy, Kusnacht, Switzerland

[b] Synacthen®, Novartis Pharma Schweiz AG, Bern, Switzerland

[c] Schellenberg S, Reusch CE, Glaus TM. Effect of adaptation on indirect blood pressure measurement in conscious untrained beagle dogs. In: Congress Proceedings 15th ECVIM-CA Congress, September 1-3, 2005, Glasgow, Scotland

[d] SDI Vet/BP 6000; SDI, Waukesha, WI

[e] Canine E.R.D.-HealthScreen® Test, Heska Corp, Fort Collins, CO

[f] Microalbumin OSR6167 Olympus system reagent, Olympus Diagnostica GmbH, Clare, Ireland

[g] Hydragel 5 Proteinurie, semiautomated Hydrasys, Sebia, Fulda, Germany

[h] Promega

[i] Thermo Finnigan

[j] SPSS 11.0 for Windows, SPSS Inc., Chicago, IL,

[k] GraphPad Prism 4, Graphpad Software Inc, San Diego, CA

[l] Jensen WA, Grauer GF, Andrews J, et al. Prevalence of microalbuminuria in dogs. In: Proceeding of the American College of Veterinary Internal Medicine annual meeting, May 23-26, 2001, Denver, CO

[m] Mazzi A, Fracassi F, Gentilini F, et al. Urinary Protein to Creatinine Ratio and Albumin to Creatinine Ratio in Dogs with Diabetes Mellitus and Pituitary Dependent Hyperadrenocorticism. In: Congress Proceedings 16th ECVIM-CA Congress, September 14-16, 2006, Amsterdam, Netherlands

REFERENCES

1 Melby JC. Clinical review 1: Endocrine hypertension. J Clin Endocrinol Metab 1989;69:697-703

2 Kelly JJ, Martin A and Whitworth JA. Role of erythropoietin in cortisol-induced hypertension. J Hum Hypertens 2000;14:195-198

3 Brotman DJ, Girod JP, Posch A, et al. Effects of short-term glucocorticoids on hemostatic factors in healthy volunteers. Thromb Res 2006;118:247-252

4 Ortega TM, Feldman EC, Nelson RW, et al. Systemic arterial blood pressure and urine protein/creatinine ratio in dogs with hyperadrenocorticism. J Am Vet Med Assoc 1996;209:1724-1729

5 Cowgill LD. Clinical significance, diagnosis, and management of systemic hypertension in dogs and cats. Partners Pract 1991;4:2-3

6 Martinez NI, Panciera DL, Abbott JA, et al. Evaluation of pressor sensitivity to norepinephrine infusion in dogs with iatrogenic hyperadrenocorticism. Pressor sensitivity in dogs with hyperadrenocorticism. Res Vet Sci 2005;78:25-31

7 Hurley KJ, Vaden SL. Evaluation of urine protein content in dogs with pituitary-dependent hyperadrenocorticism. J Am Vet Med Assoc 1998;212:369-73

8 Waters CB, Adams LG, Scott-Moncrieff JC, et al. Effects of glucocorticoid therapy on urine protein-to-creatinine ratios and renal morphology in dogs. J Vet Intern Med 1997;11:172-177

9 Feldman EC, Nelson RW. Canine and Feline Endocrinology and Reproduction, 3rd ed. St Louis, MO: WB Saunders; 2004:253-264

10 Wenger M, Sieber-Ruckstuhl NS, Muller C et al. Effect of trilostane on serum concentrations

of aldosterone, cortisol, and potassium in dogs with pituitary-dependent hyperadrenocorticism. Am J Vet Res. 2004;65:1245-1250.

11 Vincent IC, Michell AR and Leahy RA. Non-invasive measurement of arterial blood pressure in dogs: a potential indicator for the identification of stress. Res Vet Sci 1993;54:195-201

12 Gentilini F, Dondi F, Mastrorilli C, et al. Validation of a human immunoturbidimetric assay to measure canine albumin in urine and cerebrospinal fluid. J Vet Diagn Invest 2005;17:179-183

13 Keane WF, Eknoyan G. Proteinuria, albuminuria, risk, assessment, detection, elimination (PARADE): a position paper of the national kidney foundation. Am J Kidney Dis 1999 ;33:1004–1010

14 Le Bricon T, Erlich D, Bengoufa D, et al. Sodium dodecyl sulfate-agarose gel electrophoresis of urinary proteins: application to multiple myeloma. Clin Chem 1998;44:1191-1197

15 Perkins DN, Pappin DJ, Creasy DM, et al. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 1999;20:3551-3567

16 Lees GE, Brown SA, Elliott J, et al. Assessment and Management of Proteinuria in Dogs and Cats: 2004 ACVIM Forum Consensus Statement (Small Animal). J Vet Int Med 2005;19:377-385

17 Chin-Dusting JP, Ahlers BA, Kaye DM, et al. L-arginine transport in humans with cortisol-induced hypertension. Hypertension 2003;41:1336-1340

18 Goy-Thollot I, Pechereau D, Keroack S, et al. Investigation of the role of aldosterone in hypertension associated with spontaneous pituitary-dependent hyperadrenocorticism in dogs. J Small Anim Pract 2002;43:489-492

19 Stepien RL, Rapoport GS, Henik RA, et al. Comparative diagnostic test characteristics of oscillometric and Doppler ultrasonographic methods in the detection of systolic hypertension in dogs. J Vet Intern Med 2003;17:65-72

20 Brown S, Atkins C, Bagley R, et al. Guidelines for the identification, evaluation, and management of systemic hypertension in dogs and cats. J Vet Intern Med 2007; 21:542-558

21 Behrend EN, Kemppainen RJ, Boozer AL, et al. Serum 17-alpha-hydroxyprogesterone and corticosterone concentrations in dogs with nonadrenal neoplasia and dogs with suspected hyperadrenocorticism. J Am Vet Med Assoc 2005;227:1762-1767.

22 Sieber-Ruckstuhl NS, Boretti FS, Wenger M et al. Evaluation of different cortisol precursors in dogs with pituitary-dependent hyperadrenocorticism and in healthy dogs. Vet Rec accepted

23 Rijnberk A, Kooistra HS, van Vonderen IK et al. Aldosteronoma in a dog with polyuria as the leading symptom. Domest Anim Endocrinol. 2001;20:227-240

24 Connell JM, Jamieson AJ, Davies E et al. 11 beta-Hydroxylase activity in glucocorticoid suppressible hyperaldosteronism: lessons for essential hypertension? Endocr Res 1996;22:691-700

25 Remillard RL, Ross JN, Eddy JB. Variance of indirect blood pressure measurements and prevalence of hypertension in clinically normal dogs. Am J Vet Res. 1991;52:561-565

26 Schellenberg S, Glaus TM, Reusch CE. The effect of adaptation over multiple days on indirect blood pressure measurement in conscious and untrained beagle dogs. Vet Rec 2007;161: in press

27 Bodey AR, Michell AR. Epidemiological study of blood pressure in domestic dogs. J Small Anim Pract 1996;37:116-125

461 28 Hanson JM, Kooistra HS, Mol JA, Teske E, Meij BP. Plasma profiles of adrenocorticotropic
462 hormone, cortisol, alpha-melanocyte-stimulating hormone, and growth hormone in dogs with
463 pituitary-dependent hyperadrenocorticism before and after hypophysectomy. J Endocrinol
464 2006 ;190:601-609.
465 29 Silva CM, Powell-Oliver FE, Jewell CM, et al. Regulation of the human glucocorticoid
466 receptor by long-term and chronic treatment with glucocorticoid. Steroids 1994 Jul;59:436-
467 442
468 30 Knutsson U, Stierna P, Marcus C, et al. Effects of intranasal glucocorticoids on endogenous
469 glucocorticoid peripheral and central function. J Endocrinol 1995;144:301-310
470 31 Dong Y, Poellinger L, Gustafsson JA, et al. Regulation of glucocorticoid receptor expression:
471 evidence for transcriptional and posttranslational mechanisms. Mol Endocrinol 1988;2:1256-
472 1264
473 32 Burnstein KL, Bellingham DL, Jewell CM, et al. Autoregulation of glucocorticoid receptor
474 gene expression. Steroids 1991;56:52-58
475 33 Huizenga NA, De Herder WW, Koper JW, et al. Decreased ligand affinity rather than
476 glucocorticoid receptor down-regulation in patients with endogenous Cushing's syndrome.
477 Eur J Endocrinol 2000 ;142:472-476
478 34 Miki K, Morimoto T, Nose H, et al. Canine blood volume and cardiovascular function during
479 hyperthermia. J Appl Physiol 1983;55:300-306
480 35 Etxabe J and Vazquez JA. Morbidity and mortality in Cushing's disease: an epidemiological
481 approach. Clin Endocrinol (Oxf) 1994;40:479-484
482 36 Baid S, Nieman LK. Glucocorticoid excess and hypertension. Curr Hypertens Rep
483 2004;6:493-9

484 37 Sapienza P and Cavallaro A. Persistent Hypertension after removal of Adrenal Tumours. Eur
485 J Surg 1999;165:187-192
486 38 Baas JJ, Schaeffer F, Joles JA. The influence of cortisol excess on kidney function in the
487 dog. Vet Q 1984;6:17-21
488 39 Hall JE, Morse CL, Smith MJ et al. Control of arterial pressure and renal function during
489 glucocorticoid excess in dogs. Hypertension 1980;2:139-148.
490 40 Kubota E, Hayashi K, Matsuda H et al. Role of intrarenal angiotensin II in glucocorticoid-
491 induced renal vasodilation. Clin Exp Nephrol 2001;5:186-192
492 41 Koh JM, Kim JY, Chung YE, et al. Increased urinary albumin excretion in Cushing's
493 syndrome: remission after correction of hypercortisolaemia. Clin Endocrinol (Oxf)
494 2000;52:349-353
495 42 Gary AT, Cohn LA, Kerl ME, et al. The effects of exercise on urinary albumin excretion in
496 dogs. J Vet Intern Med 2004;18:52-55
497 43 Dyer AR, Greenland P, Elliott P, et al. Evaluation of measures of urinary albumin excretion
498 in epidemiologic studies. Am J Epidemiol 2004;160:1122-1131
499 44 Hansen HP, Hovind P, Jensen BR, et al. Diurnal variations of glomerular filtration rate and
500 albuminuria in diabetic nephropathy. Kidney Int 2002;61:163-168
501 45 van Acker BA, Stroomer MK, Gosselink MA, et al. Urinary protein excretion in normal
502 individuals: diurnal changes, influence of orthostasis and relationship to the renin-
503 angiotensin system. Contrib Nephrol 1993;101:143-150
504 46 46 Jensen JS, Feldt-Rasmussen B, Borch-Johnsen K et al. Microalbuminuria and its relation
505 to
506 cardiovascular disease and risk factors. A population-based study of 1254 hypertensive

507 individuals. J Hum Hypertens 1997;11:727-732.
508 47 Bigazzi R, Bianchi S, Baldari D et al. Microalbuminuria predicts cardiovascular events and
509 renal insufficiency in patients with essential hypertension. J Hypertens 1998;16:1325-1333.
510 48 Yudkin JS, Forrest RD, Jackson CA. Microalbuminuria as predictor of vascular disease in
511 non-diabetic subjects. Islington Diabetes Survey. Lancet. 1988;2:530-533.
512 49 Kuusisto J, Mykkanen L, Pyorala K et al. Hyperinsulinemic microalbuminuria. A new risk
513 indicator for coronary heart disease. Circulation 1995;91:831-837.
514 50 Chapdelaine P, Dube JY, Frenette G, et al. Identification of arginine esterase as the major
515 androgen-dependent protein secreted by dog prostate and preliminary molecular
516 characterization in seminal plasma. J Androl 1984;5:206-210
517 51 Isaacs WB, Coffey DS. The predominant protein of canine seminal plasma is an enzyme. J
518 Biol Chem 1984;259:11520-11526
519 52 Bonfanti U, Zini E, Minetti E, et al. Free light-chain proteinuria and normal renal
520 histopathology and function in 11 dogs exposed to Lleishmania infantum, Ehrlichia canis,
521 and Babesia canis. J Vet Intern Med 2004;18:618-624

LEGENDS

Fig 1. Change in urine protein:creatinine ratios (UPC) in 12 dogs before, during and after administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant difference between the different time points. The grey background indicates the hydrocortisone or placebo treatment period.

Fig 2. Change in urine albumin:creatinine ratios (UAC) in 12 dogs before, during and after administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant difference between the different time points. The grey background indicates the hydrocortisone or placebo treatment period.

Fig 3. Analysis of urine protein patterns by agarose gel electrophoresis followed by staining with acid violet. The different protein patterns found in urine samples obtained from dogs in the control group (A). The change in urine protein pattern in a hydrocortisone treated female dog at the different time points (B). M, marker; m, male dog; f, female dog; Lxy, Lane xy

Fig 4. Representative MSMS spectrum of the tryptic peptide AVIRPGEDR of canine ariginine esterase (ESTA_CANFA) identified by Mascot. The major fragments generated are labeled and the y- and b- fragments detected are indicated in the sequence.

Table 1. Median values and ranges of systolic (SAP), diastolic (DAP) and mean arterial blood pressure (MAP) in control and hydrocortisone treated dogs at the different time points.

	SAP (mmHg)		DAP (mmHg)		MAP (mmHg)	
	Controls Median (Range)	**I-HAC** Median (Range)	**Controls** Median (Range)	**I-HAC** Median (Range)	**Controls** Median (Range)	**I-HAC** Median (Range)
d0	128 (122 - 142)	123 (114 - 136)	63 (55 - 83)	69 (53 - 78)	90 (78 - 104)	90 (81 - 101)
d1	128 (123 - 135)	130 (118 - 136)	68 (63 - 75)	70 (66 - 78)	89 (82 - 95)	91 (84 - 99)
d5	126 (117 - 148)	133 (122 - 144)	66 (56 - 77)	75 (64 - 81)	89 (80 - 98)	94 (82 - 103)
d28	129 (116 - 149)	143 (128 - 148)[a]	70 (65 - 83)	81 (77 - 90)[a]	94 (83 - 107)	102 (95 - 109)[a]
d56	130 (122 - 145)	137 (134 - 139)[a]	72 (63 - 86)	82 (74 - 82)[a]	96 (84 - 105)	103 (96 - 105)[a]
d84	127 (119 - 144)	131 (129 - 141)	66 (59 - 81)	80 (71 - 81)[a]	89 (80 - 106)	98 (94 - 104)[a]
d1p	129 (118 - 141)	130 (124 - 144)	66 (54 - 75)	78 (65 - 84)	91 (80 - 103)	98 (89 - 103)
d5p	133 (117 - 152)	124 (106 - 136)	73 (53 - 86)	70 (55 - 76)	94 (77 - 115)	88 (77 - 98)
d28p	131 (123 - 145)	124 (117 - 128)	66 (60 - 86)	69 (61 - 81)	91 (86 - 105)	87 (84 - 97)
d56p	126 (118 - 140)	124 (116 - 130)	70 (59 - 81)	70 (61 - 82)	91 (85 - 104)	87 (79 - 101)
d84p	130 (121 - 143)	127 (117 - 133)	67 (60 - 79)	68 (62 - 77)	89 (79 - 107)	91 (85 - 95)

dx, day x of hydrocortisone treatment, dxp, day x after hydrocortisone treatment

[a] $p < 0.05$ versus d0

Table 2. Median values, ranges and P-values (control vs. I-HC dogs) of urine protein:creatinine (UPC) and urine albumin:creatinine (UAC) ratios in control dogs and dogs of the I-HC group at the different time points.

	UPC			UAC		
	control dogs Median (Range)	**I-HC dogs** Median (Range)	**P value**	**control dogs** Median (Range)	**I-HC dogs** Median (Range)	**P value**
d0	0.21 (0.13 - 0.38)	0.17 (0.15 - 0.28)	0.937	0.017 (0.005 - 0.082)	0.015 (0.009 - 0.023)	0.937
d1	0.15 (0.12 - 0.32)	0.12 (0.09 - 0.22)	0.240	0.014 (0.006 - 0.060)	0.013 (0.010 - 0.028)	0.818
d5	0.18 (0.10 - 0.23)	0.15 (0.08 - 0.28)	0.937	0.013 (0.004 - 0.048)	0.016 (0.005 - 0.043)	1.000
d28	0.19 (0.13 - 0.29)	0.38 (0.18 - 1.78)[a]	0.026	0.024 (0.004 - 0.067)	0.045 (0.012 - 0.795)	0.240
d56	0.19 (0.11 - 0.30)	0.30 (0.20 - 0.51)	0.041	0.016 (0.005 - 0.079)	0.083 (0.021 - 0.339)[b]	0.015
d84	0.22 (0.10 - 0.36)	0.33 (0.21 - 1.63)[a]	0.065	0.020 (0.003 - 0.103)	0.133 (0.034 - 1.251)[b,c]	0.026
d1p	0.24 (0.09 - 0.34)	0.34 (0.20 - 1.03)	0.180	0.023 (0.003 - 0.070)	0.101 (0.011 - 0.725)	0.041
d5p	0.24 (0.12 - 0.30)	0.24 (0.19 - 0.67)	0.699	0.035 (0.002 - 0.078)	0.037 (0.016 - 0.311)	0.589
d28p	0.20 (0.08 - 0.36)	0.17 (0.11 - 0.31)	0.937	0.020 (0.002 - 0.093)	0.010 (0.005 - 0.032)	1.000
d56p	0.18 (0.09 - 0.36)	0.18 (0.14 - 0.22)	0.937	0.018 (0.004 - 0.113)	0.013 (0.003 - 0.025)	0.699
d84p	0.25 (0.10 - 0.35)	0.20 (0.13 - 0.30)	0.699	0.027 (0.007 - 0.104)	0.014 (0.005 - 0.037)	0.485

dx, day x of hydrocortisone treatment, dxp, day x after hydrocortisone treatment

[a] $p < 0.05$ versus d1 and d5, [b] $p < 0.05$ versus d1, [c] $p < 0.05$ versus d0

Table 3. Median values, ranges and P-values (male vs female dogs) of urine protein:creatinine ratios (UPC) in all male and female dogs at the different time points.

	UPC		
	male dogs Median (Range)	**female dogs** Median (Range)	**P value**
d0	0.28 (0.18 - 0.38)	0.15 (0.13 - 0.16)	0.002
d1	0.19 (0.11 - 0.32)	0.13 (0.09 - 0.15)	0.041
d5	0.22 (0.16 - 0.28)	0.12 (0.08 - 0.14)	0.002
d28	0.30 (0.23 - 1.78)	0.17 (0.13 - 1.63)	0.065
d56	0.28 (0.20 - 0.49)	0.20 (0.11 - 0.51)	0.394
d84	0.33 (0.21 - 1.63)	0.24 (0.10 - 0.34)	0.180
d1p	0.34 (0.20 - 1.03)	0.20 (0.09 - 0.39)	0.065
d5p	0.29 (0.19 - 0.67)	0.19 (0.12 - 0.28)	0.026
d28p	0.30 (0.12 - 0.36)	0.11 (0.08 - 0.17)	0.009
d56p	0.22 (0.16 - 0.36)	0.14 (0.09 - 0.19)	0.004
d84p	0.29 (0.21 - 0.35)	0.14 (0.10 - 0.21)	0.002

dx, day x of hydrocortisone treatment, dxp, day x after hydrocortisone treatment

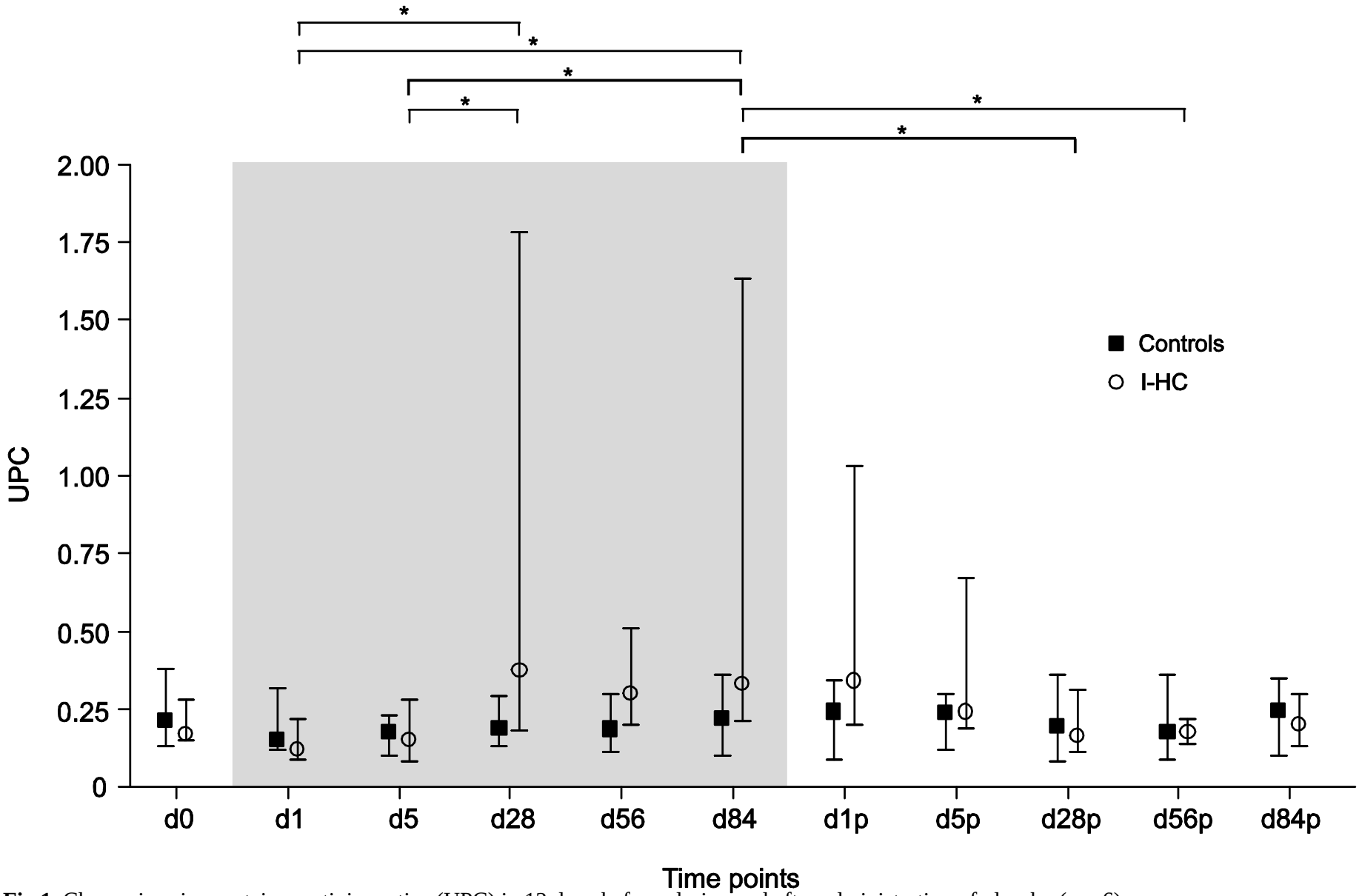


Fig 1. Change in urine protein:creatinine ratios (UPC) in 12 dogs before, during and after administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant difference between the different time points. The grey background indicates the hydrocortisone or placebo treatment period.

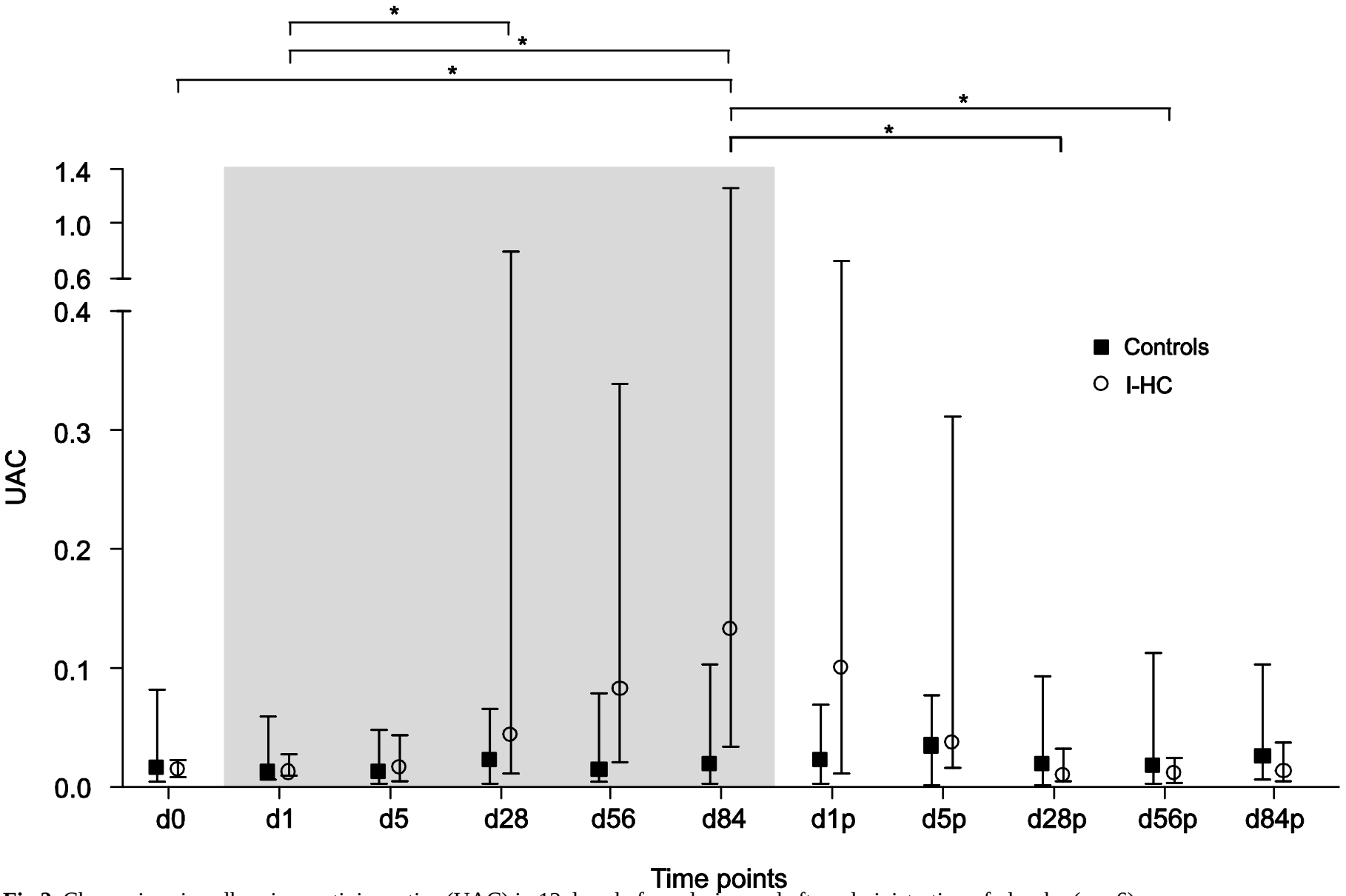
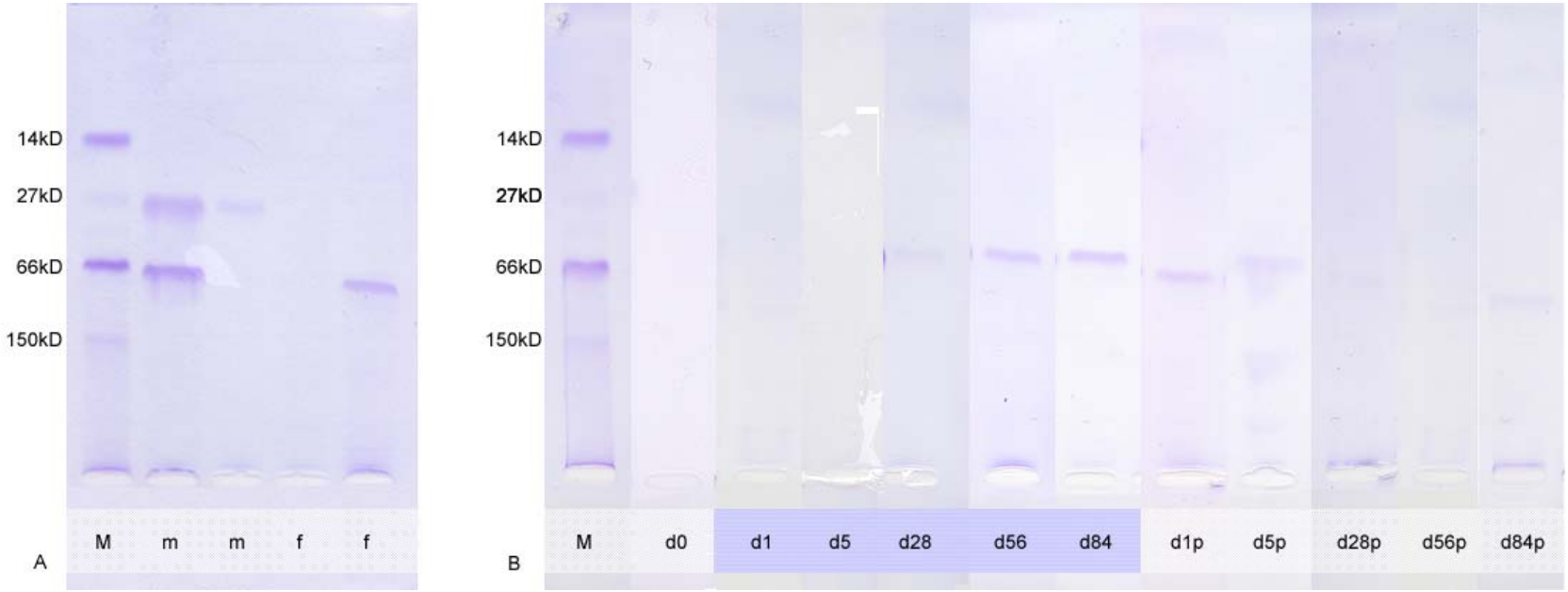


Fig 2. Change in urine albumin:creatinine ratios (UAC) in 12 dogs before, during and after administration of placebo (n = 6) or hydrocortisone (n = 6). An asterisk (*) indicates significant difference between the different time points. The grey background indicates the hydrocortisone or placebo treatment period.



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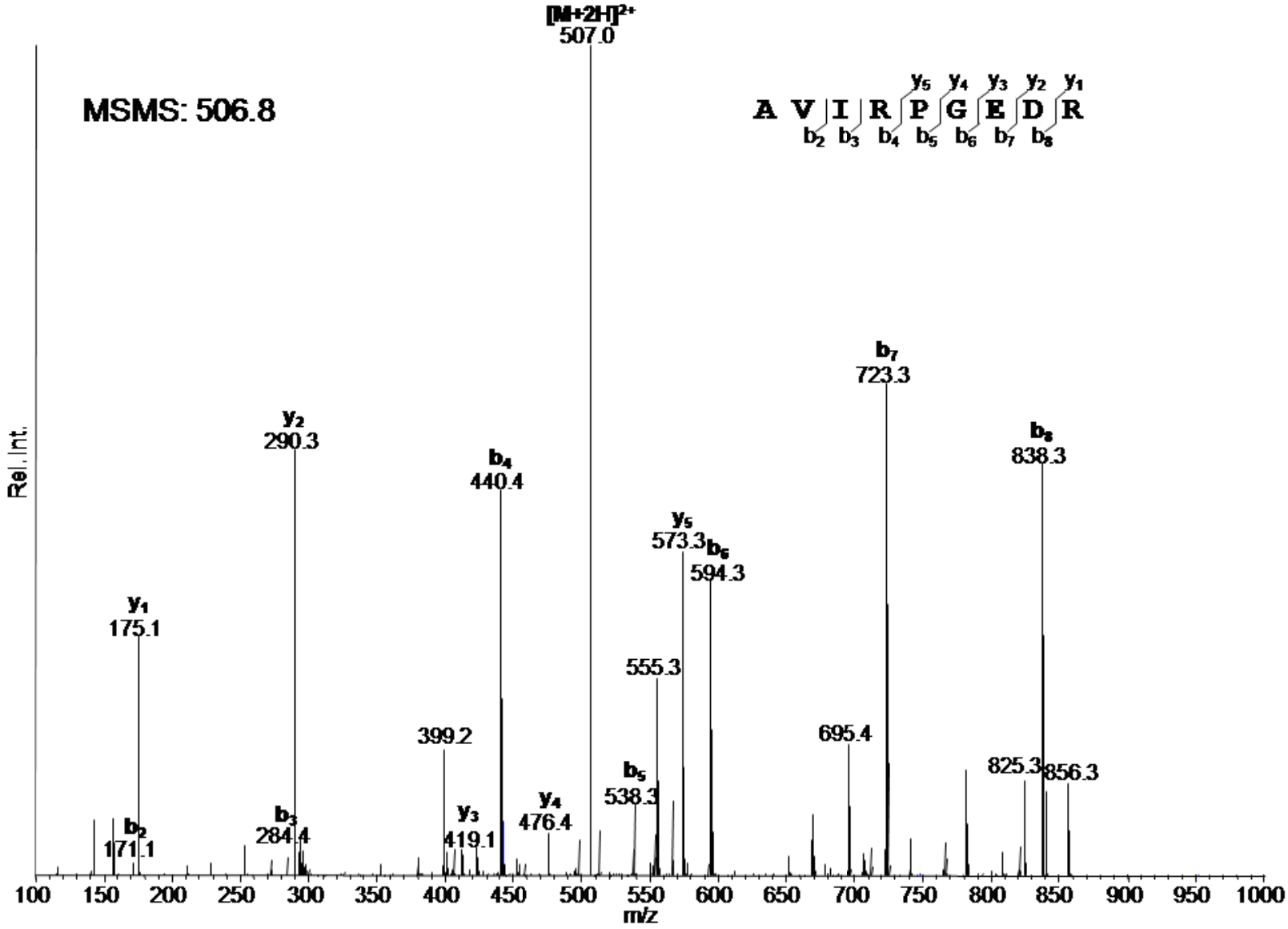

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